CRISPR/Cas-induced targeted mutagenesis with Agrobacterium mediated protein delivery

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Chapter 3

Targeted mutagenesis in yeast with the Cas9 protein translocated through the type IV secretion system of *Agrobacterium*

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Abstract

The RNA guided Cas9 endonuclease derived from the type II CRISPR/Cas system from *Streptococcus pyogenes* has been developed as a new potent tool for gene knockout in bacteria, yeast, fungi, animal cells and plants. Usually the system is introduced in cells by transfer of the encoding genes. Here we show that the Cas9 protein can also be delivered in cells through the bacterial type IV secretion system (T4SS) of the plant pathogen *Agrobacterium tumefaciens*. Cas9 transfer was effectuated by fusion of a T4SS translocation peptide to the Cas9 protein. Co-cultivation of yeast with an *Agrobacterium* strain expressing both Cas9 and the sgRNA did not lead to mutations in yeast. However after co-cultivation of a yeast expressing the sgRNA for CAN1 (canavanine) with an *Agrobacterium* strain expressing Cas9 (CAN1) mutations were obtained. Concurrent translocation of a T-DNA together with the Cas9 protein was possible and among T-DNA transformations CAN1 mutants could be identified at low frequency. However, concurrent translocation of a T-DNA encoding the sgRNA and Cas9 did not result in targeted mutations at the CAN1 locus.

Introduction

The RNA guided endonucleases encoded by the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) systems are of great interest to the biotechnology community because of their use in genome editing. In prokaryotes where the CRISPR/Cas system originates, it functions as an adaptive immune system that acts against bacteriophages and other invading nucleic acids [1]. The Cas9 protein from the type II bacterial CRISPR/Cas system found in *Streptococcus pyogenus*, can be programmed by an engineered single guide RNA (sgRNA) to create targeted double strand breaks (DSBs) at a desired sequence in any host cell [2]. By specifying 20 nucleotides of the sgRNA the Cas9 endonuclease can be directed to 20 basepair complementary target sequences [2,3]. A limitation is that the target sequence must be flanked by a protospacer-associated motif (PAM) that is required for Cas9 activity [4,5]. DSBs introduced in the genome with the CRISPR/Cas system can either be repaired via non-homologous end joining (NHEJ) or by homologous recombination (HR). Repair via the error prone NHEJ pathway can result in small deletions and insertions enabling effective reverse genetics. Repair via HR can be used to replace or correct existing genes by providing an artificial repair template with homology to the target sequence. For targeted mutagenesis of host cells the genes encoding the Cas9 nuclease and the sgRNA are usually introduced in the new host and expressed. High expression levels of these transgenes is however not wanted because it increases the frequency of off-target mutations [6,7]. Here, we developed a system for the direct delivery of the Cas9 protein to yeast through the type IV secretion system of Agrobacterium tumefaciens.

This bacterium is a soil-dwelling organism that is most commonly known for causing crown gall disease in plants by genetically transforming these with a set of oncogenes. It is the preferred vector for plant transformation, but is also frequently used as a vector for the transformation of yeasts [8] and fungi [9,10]. *Agrobacterium* uses a Type IV Secretion System (T4SS), encoded by the *virB* genes and *virD4* gene on its Ti plasmid, for the translocation of the T-DNA [11–13]. Along with the T-DNA, several virulence proteins are translocated independently of the T-DNA into the host cell [14]. Translocation of proteins through the T4SS has been shown to be dependent on a hydrophilic C-terminal secretion signal with a net positive charge [15]. Certain heterologous proteins can be translocated through the T4SS

after fusion to the translocation signal of one of the virulence proteins [14,16,17]. In this way the Cre recombinase and homing endonuclease I-SceI were translocated by *Agrobacterium* into host cells to effect DNA recombination in the genome of target cells [14–16]. Here, we developed a system for the direct delivery of the Cas9 protein to yeast through the type IV secretion system of *Agrobacterium*.

Results

Translocation of the Cas9 endonuclease

To test if the Cas9 protein could be translocated through the T4SS of *Agrobacterium*, an expression plasmid was created encoding a Cas9 fusion protein with a N-terminal nuclear localization signal and the C-terminal 37 amino acid translocation signal of the *Agrobacterium* virulence protein VirF (pNCas9F). The production of the fusion protein (NCas9F) was under control of the acetosyringone inducible *virF* promoter to ensure that production would only occur in the presence of a functional T4SS. The CAN1 gene was used as target locus for double strand break induction by Cas9. As an eukaryotic recipient we used a yeast strain expressing a sgRNA targeting the CAN1 locus from the strong TEF1 promoter [18]. This locus encodes a plasma membrane arginine transporter which mediates the uptake of arginine and its toxic analogue L-canavanine into the cell. Mutation of CAN1 leads to L-canavanine resistance. In our experiments translocation of NCas9F could therefore be detected by an increased number of L-canavanine resistant cells after co-cultivation with *Agrobacterium*.

The CAN1 mutation frequency of the recovered yeast cells was found to be ~2.5 * 10^{-5} after co-cultivation with *Agrobacterium* strain LBA1100 expressing NCas9F compared to ~0.2 * 10^{-5} after control co-cultivation with the same *Agrobacterium* lacking the NCas9F expression plasmid. To show that this about tenfold increase in the frequency of L-canavanine resistant colonies was the result of the combined nuclease activity of the translocated NCas9F and the CAN1 sgRNA, the CAN1 locus of 16 L-canavanine resistant colonies obtained after co-cultivation was amplified by PCR and Sanger sequenced. This revealed that 15 of the 16 L-canavanine resistant colonies had mutations directly upstream of the PAM sequence (Fig. 2a), whereas the CAN1 locus of the 16th colony had a single base pair substitution 300 bp upstream of the PAM sequence. The eight L-canavanine resistant colonies obtained after a co-cultivation with an *Agrobacterium* strain lacking NCas9F had mutations that were not located directly upstream of the PAM sequence but were instead randomly distributed throughout the CAN1 gene and therefore represented spontaneous mutations.

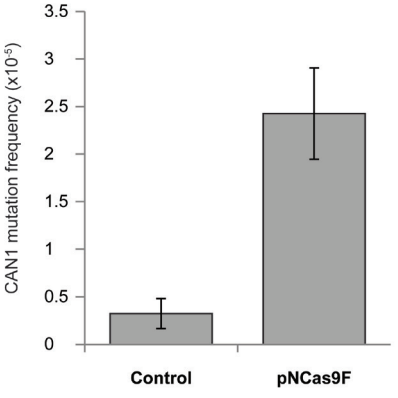

Figure 1. Translocated Cas9 mediated mutagenesis. CAN1 mutation frequency found in yeast after co-cultivation with an *Agrobacterium* strain translocating NCas9F (pNCas9F) and an *Agrobacterium* strain lacking NCas9F (Control). Error bars indicate the SEM (N=3).

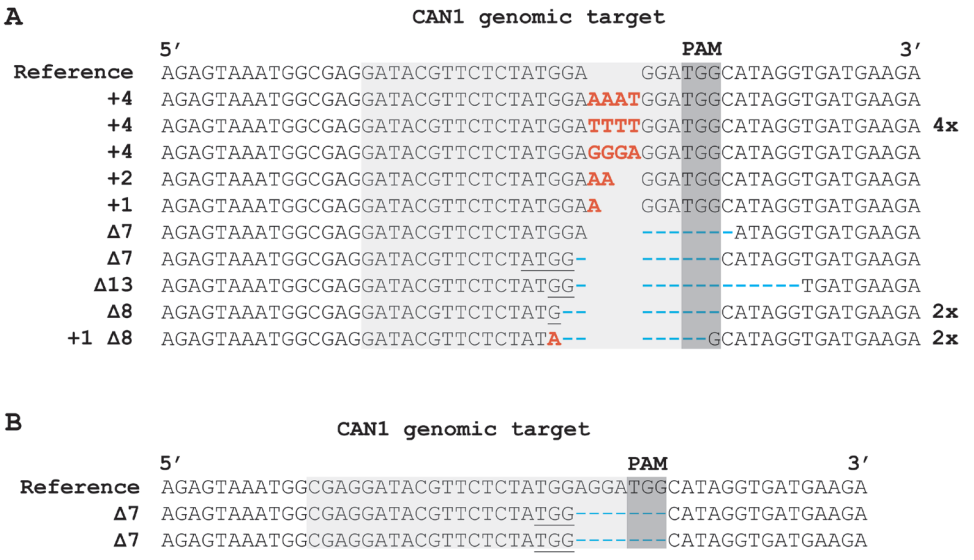

Figure 2. Alignments of the CAN1 target with sequences obtained from L-canavanine resistant colonies after co-cultivation. The PAM sequence is highlighted in dark grey and the recognition sequence of the sgRNA is in light grey. Insertions are marked in red and deletions are marked in blue and microhomologies are underlined. The occurrence of the mutations is noted on the right hand side. (**A**) Mutations found after co-cultivation with an *Agrobacterium* strain delivering the NCas9F protein. (**B**) Mutations found after co-cultivation with an *Agrobacterium* strain delivering both NCas9F and a T-DNA mediating G418 resistance.

Translocation of NCas9F and sgRNA by Agrobacterium

As the previous experiments showed that the NCas9F was functional after translocation we performed an experiment to test the translocation of a NCas9F and a sgRNA complex through the T4SS of *Agrobacterium*. A plasmid was created that puts CAN1 sgRNA production under the control of the *virF* promoter and has the 3' flanking region of *virF* functioning as a terminator sequence (psgRNAvirF5'). The functionality of the *Agrobacterium* sgRNA expression cassette was confirmed with an assay in which DSBs induced by the sgRNA/NCas9F complex stimulated the looping out of a previously plasmid integrated into the genome of *Agrobacterium* (Chapter 2). The frequency of L-canavanine resistant colonies was determined after co-cultivation with several different *Agrobacterium* strains that expressed either the combination of both the sgRNA and NCas9F, neither one or only NCas9F (Fig. 3). A comparison between the different CAN1 mutation frequencies showed the expression of sgRNA in *Agrobacterium* did not enhance the mutation frequency, and that co-cultivations with translocated NCas9F only led to an increased mutation frequency if the sgRNA was expressed in yeast cells. Co-cultivation with the yeast strain expressing the CAN1 sgRNA confirmed that NCas9F protein was translocated from *Agrobacterium* in the presence of sgRNA. These results therefore indicate that sgRNA/NCas9F complexes are not translocated by the T4SS.

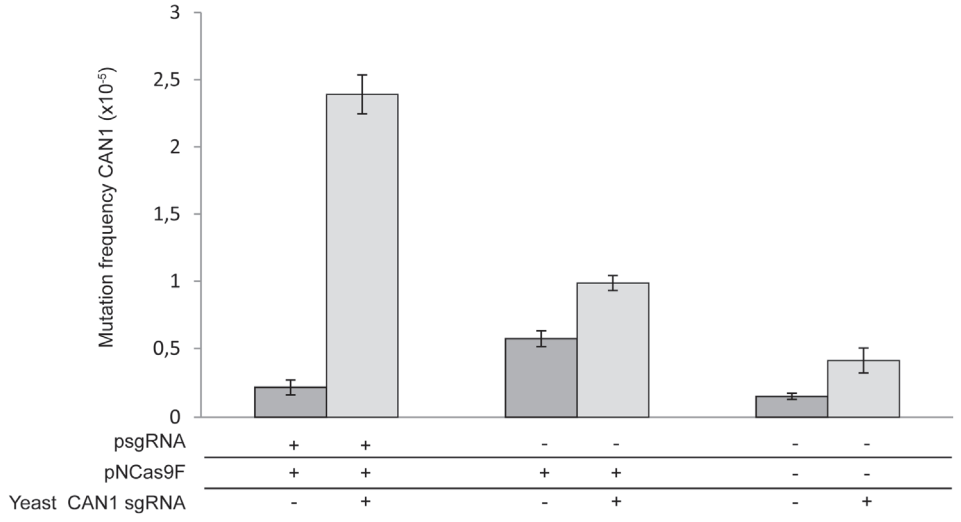

Figure 3. Translocation of sgRNA/NCas9F complex. CAN 1 mutation frequencies after co-cultivation with *Agrobacterium* translocating NCas9F (pNCas9F) in the presence or absence of sgRNA production in (psgRNA) in *Agrobacterium*. Bottom row indicates the presence of the sgRNA targeting the CAN1 in the recipient yeast strain. Error bars indicate the SEM (N=3).

Comparison of mutagenesis by translocated Cas9 protein and Cas9 expressed from a translocated T-DNA

To compare the targeted mutagenesis frequencies between translocated Cas9 and Cas9 from a translocated T-DNA two T-DNA vectors were created both containing an expression cassette in which the NCas9F protein is expressed from the strong TEF1 promoter. The first T-DNA vector pNCas9FPDA1 contains a T-DNA that upon entry of a yeast cell can integrate into the yeast genome at the PDA1 locus via homologous recombination. The second T-DNA vector contains a T-DNA with a 2μ origin of replication that circularizes and replicates in yeast (pNCas9F2μ). Both of these T-DNA vectors carry a dominant KanMX selectable marker which allows selection of transgenic yeast resistant to G418. Immediately after co-cultivation with a recipient yeast expressing a sgRNA directed to the CAN1 locus the mutation frequency was determined by selection on L-canavanine. The results (Fig. 4) obtained indicated that co-cultivation with an *Agrobacterium* strain translocating NCas9F results in a CAN1 mutation frequency that is in the same order of magnitude as the mutation frequency after co-cultivation with either of the strains delivering a T-DNA from which NCas9F is expressed.

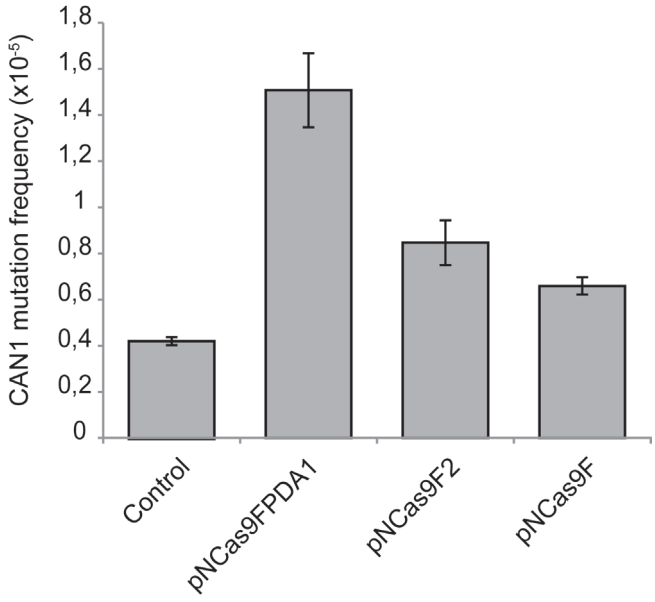

Figure 4. Translocated NCas9F compared to constitutively expressed NCas9F. Mutation frequencies of CAN1 (x10-5) after co-cultivation of a recipient yeast expressing the CAN1 sgRNA with *Agrobacterium* donor strains with integrative T-DNA vector expressing NCas9F (pNCas9FPDA1), circularizing T-DNA vector expressing NCas9F (pNCas9F2µ) or protein translocation vector with NCas9F (pNCas9F). Error bars indicate the SEM (N=3).

Recovery frequency of mutants at non-selectable loci

To determine at which frequency yeast colonies with mutations at non-selectable loci could be recovered by our methodology, an *Agrobacterium* strain was created that not only translocates the NCas9F protein but also a T-DNA conferring G418 resistance (pSDM8002). This T-DNA was used to determine the frequency at which mutated yeast colonies could be recovered for non-selectable loci.

A co-cultivation was conducted with this *Agrobacterium* strain and a yeast strain expressing the sgRNA targeting the CAN1 locus. After co-cultivation yeast transformants were selected on G418 containing medium, and the G418 resistant colonies were subsequently tested for L-canavanine resistance. Out of a total of 1890 transformants, two colonies from independent co-cultivations were found that were also L-canavanine resistant. Sequencing of the CAN1 locus revealed that both colonies had the same 7 basepair deletion upstream of the PAM (Fig. 2b) that was previously found already in the first experiment. These results indicate that after NCas9 translocation targeted mutations can be recovered in yeast at non-selectable loci at a frequency of about 1:1000 T-DNA transformants.

Translocation of both Cas9 and a T-DNA expressing the sgRNA

As we could not obtain evidence for translocation of the complete NCas9F complex from *Agrobacterium* into yeast, we subsequently studied whether we could obtain targeted mutagenesis in yeast by translocation of the NCas9F protein combined with the translocation of a T-DNA coding for the sgRNA could induce targeted mutations. For this purpose two sgRNA expression vectors were created, an integrative T-DNA vector (psgRNAPDA1) and a

circularizing T-DNA vector (psgRNA2µ) both conferring to G418 resistance. Co-cultivations were performed with *Agrobacterium* strains expressing NCas9F in combination with either of the sgRNA encoding T-DNA vectors. These co-cultivations did however not increase the CAN1 mutation frequency (Fig. 5). Transfer of both T-DNA's was confirmed by selection on G418. The transfer of the NCas9F protein in the presence of either T-DNA vector was confirmed when the co-cultivations were performed with a recipient yeast strain expressing the CAN1 sgRNA (Fig. 5). Because previous experiments showed that the concurrent transfer of the NCas9F protein and a T-DNA occurs at low frequencies, the same co-cultivations were performed at a much larger scale and a total of $2*10^9$ recovered yeast cells was plated on dual selection medium containing both L-canavanine and G418. No colonies were however found that were L-canavanine resistant due to mutations directly upstream of the PAM. These results combined indicate that concurrent transfer of the NCas9F protein and a T-DNA expressing the sgRNA did not induce targeted mutations.

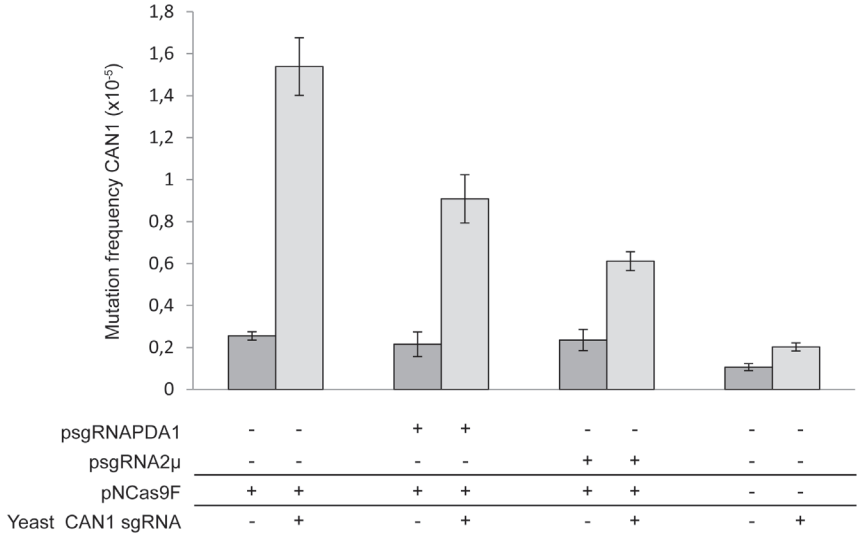

Figure 5. Mutation frequency after co-cultivation with integrative T-DNA expressing the CAN1 sgRNA (psgRNAPDA1), circularizing T-DNA expressing the CAN1 sgRNA (psgRNA2µ) and translocated NCas9F (pNCAS9F). CAN1 mutation frequencies after co-cultivation with yeast strain not expressing the sgRNA (dark grey) and a yeast strain expressing the sgRNA (light grey). Bottom row indicates the presence of the sgRNA targeting the CAN1 in the recipient yeast strain. Error bars indicate the SEM (N=3).

Discussion

In this study we have shown that it is possible to translocate Cas9 of the type II bacterial CRISPR system from *Agrobacterium* to yeast. After translocation, Cas9 was capable of forming a complex with a sgRNA and create targeted DSBs that after imperfect repair via NHEJ resulted in targeted mutations. Several of the mutations found within the CAN1 gene were probably the result of repair using small existing 4bp microhomologies (Fig. 2a and Fig. 2b). This may be one of the preferred ways to repair the DSB in the absence of a repair template [19–21].

The mutation frequency which we found with translocated NCas9F is about a tenfold lower than the previously reported mutation frequencies obtained when Cas9 was expressed directly from a plasmid in yeast [18]. This could be due to limiting levels of translocated NCas9F, the short presence of NCas9F in the host after translocation or because not every yeast cell in the co-cultivation mixture is susceptible to *Agrobacterium* mediated transfer of NCas9F, or could be the consequence of a combination of these factors.

Mutations induced by the translocation of the sgRNA/NCas9F complex from *Agrobacterium* to yeast could not be detected in the experiments performed. Mutations were however found if an *Agrobacterium* strain expressing both NCas9F and the sgRNA was co-cultivated with a yeast strain expressing the sgRNA, confirming the translocation of NCas9F in the presence of the sgRNA. Furthermore we showed that the sgRNA/NCas9F complex forms a functional complex in *Agrobacterium* (Chapter 2). Therefore we speculate that the large size of the NCas9F/sgRNA complex (100 Å x 100 Å x 50 Å) [22] and other large proteins probably requires (partial) unfolding to be transferred complex through the T4SS pore which is only 10 Å at its narrowest point [23]. Such unfolding would disrupt the interaction between NCas9F and the sgRNA.

The mutation frequencies found with translocated NCas9F protein and Cas9 expressed from a T-DNA were in the same order of magnitude. This showed that protein translocation of Ncas9F occurs at a similar frequency as T-DNA transfer. Transient presence of NCas9F could have a positive effect on the frequency of off-target mutations by the limiting availability of the nuclease [6,7].

Combined transfer of NCas9F and a T-DNA encoding the CAN1 sgRNA did not result in targeted mutations after co-cultivation, although transfer of both NCas9F and the T-DNA could be detected individually. Combined transfer of NCas9F and a T-DNA encoding the sgRNA is however capable of inducing targeted mutations in plants after ten days (unpublished data). Therefore we speculate that the lack of targeted mutations is caused by insufficient levels of sgRNA, or because sufficient levels of sgRNA requires the double strand formation of the T-DNA expressing the sgRNA at which point the translocation of NCas9F has potentially already stopped.

In summary the experimental data discussed above show that it is possible to use *Agrobacterium* for the translocation of the Cas9 protein into yeast cells to create targeted mutations in yeasts. Because under laboratory conditions *Agrobacterium* is capable of transforming various other hosts including filamentous fungi, our methodology may be applicable more broadly.

Material and methods

Yeast strains and media

Derivatives of the *Saccharomyces cerevisiae* strain YPH499 (MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1) were used in all experiments. YPH499 was grown on YEPD before transformation and then propagated on SD medium minus the auxotrophic compound(s) complemented by the plasmid(s). Transformation of YPH499 was done using the standard LiAc method [24].

Agrobacterium strains

The *Agrobacterium* strain LBA1100 (C58 containing pTiB6Δ (ΔT-DNA, Δocc, Δtra), Rif, Spc) was used for all experiments [25]. *Agrobacterium* was grown in LB (5 g/l NaCl) medium containing the appropriate antibiotics at the following concentrations: gentamicin 40 μg/ml, rifampicin 10 μg/ml, spectinomycin 250 μg/ml, kanamycin 100 μg/ml. Plasmids were electroporated to LBA1100 as described in den Dulk-Ras and Hooykaas (1995).

Plasmid construction

For the construction of pNCas9F see Chapter 2. The pSDM8002 backbone was created by cloning the 2μ replicon EcoRI fragment from pSLF178k [28] into the EcoRI site of pSDM8000 [29]. To create the T-DNA vectors that expres NCas9F, the NLS::Cas9F::VirF fragment was amplified via PCR with DS122 and DS123. This PCR fragment was digested with XbaI, and cloned into the XbaI and blunted (Klenow) XhoI site of p414-TEF1p-Cas9-CYC1t (Addgene #43802, [18]). From the resulting backbone the NLS::Cas9::VirF including the 5' TEF1 promoter and the 3' CYC1 terminator was amplified by PCR with DS124 and DS125. This PCR fragment was cloned into the blunted XbaI site (Klenow) of pSDM8001 [30] to create pNCas9FFPDA1 and the blunted XhoI site of pSDM8002 to create pNCas9F2μ. The pSDM8002 backbone was created by cloning the 2μ replicon EcoRI fragment from pSLF178k [28] into EcoRI site of pSDM8000 [29]. The T-DNA sgRNA expression vectors were created by amplifying the CAN1 sgRNA expression cassette including the SNR52 promoter and the SUP4 terminator from p426-SNR52p-gRNA.CAN1.Y-SUP4t (Addgene #43803, [18]) via PCR with either DS114 and DS115 or DS119 and DS120 and inserted into the XbaI site of pSDM8001 and the XhoI site of pSDM8002, respectively.

To create the *Agrobacterium* sgRNA expression vector, the *virF* 3' region was amplified using DS097 and DS099 adding a XbaI, StuI and SalI site and two flanking BglII sites. The resulting PCR fragment was cloned into the BglII site pOPHIS Borderless [31]. The *virF* 5' flanking region was amplified using DS100 and DS101 and inserted into the XbaI and StuI sites of pOPHIS with the *virF* 3' flanking region. The complementary oligo's DS104 and DS105 were annealed and inserted into the BsbI sites of pEN_Chimera [32]. From pEN_Chimera the entire CAN1 sgRNA cassete was amplified by PCR with DS102 and DS103 and cloned into the StuI and SalI sites of pOPHIS with the *virF* 5' and *virF* 3' flanking regions creating psgRNA.

Co-cultivations of Agrobacterium with yeast

Co-cultivations of *Agrobacterium* with the YPH499 yeast strain were carried out as previously described with the following minor modifications [33]. *Agrobacterium* was grown overnight at 29°C in LB (5 g/l NaCl) with appropriate antibiotics. Induction of the *virF* promoter was performed at 28°C at OD_{600} = 0.25 for 6 hours in induction medium [30] containing 200 μM acetosyringone (Sigma-Aldrich Co.). The yeast strain with the p426-SNR52p-gRNA.CAN1.Y-SUP4t plasmid [18] was grown overnight in MY minus the auxotrophic growth compound (uracil) complemented by the plasmid and then diluted 10 times in YEPD and cultured for 6 hours. Yeast (10^7 cells) and *Agrobacterium* ($2*10^8$ cells) were mixed and spotted on nitrocellulose filters on induction medium plates [8] containing 200μM acetosyringone (Sigma-Aldrich Co.) followed by an incubation at 21°C for 7 days.

Determination of the CAN1 mutation frequency and T-DNA transfer frequency

Yeast was recovered from the nitrocellulose filter and plated on minimal yeast medium containing L-canavanine sulfate (60 μg mL^{-1}, Santa Cruz Biotechnology Inc.) to select for mutations of the CAN1 locus and cefotaxime (200 μg mL^{-1}, FORMEDIUM™) to stop the growth of *Agrobacterium*. Total yeast cell numbers were determined by plating serial dilutions of yeast on YEPD plates containing cefotaxime (200 μg mL^{-1}, FORMEDIUM™). The CAN1 mutation frequency was determined by dividing the number of L-canavanine resistant colonies by total colony count based on the serial dilutions on YEPD. To determine the T-DNA transfer frequency yeast recovered from the nitrocellulose filter was plated on YEPD containing G418 (200 μg mL^{-1}, FORMEDIUM™) and cefotaxime (200 μg mL^{-1}, FORMEDIUM™). The transfer frequency was determined by dividing the number of G418 resistant colonies by the total colony count based on the serial dilutions on complete yeast medium.

Mutation analysis of the CAN1 locus

Yeast was grown overnight in YEPD at 30°C. Genomic DNA was isolated from 2 ml cultures using the yeastar^{tm} genomic DNA kit from Zymoclean (protocol 1). The CAN1 locus was amplified from L-canavanine resistant colonies by PCR with primers DS086 and DS088 and the PCR fragment was cloned into pJET2.1 (CloneJET PCR Cloning Kit, Thermo Fischer Inc.) before sequencing (Macrogen Europe Inc.).

Table 1. Overview of primers used in this study

Primer name	Sequence
DS060	GATCTACTAGTGCTGCACGG
DS061	GATCCCGTGCAGCACTAGTA
DS072	AGTCAGATCTGAAAAACATCAAAAAAAACCAG
DS073	GCTAAGATCTCGTTTCAGGAAAGTTTCGGAGGAG
DS086	CTTCAGACTTCTTAACTCCTGT
DS088	TGAGGGTGAGAATGCGAAATG
DS097	AGGCCTCCTTCCGTCGACCTATCCGTGCTGTTCGTCAC
DS098	AGATCTTCTAGACCTTCCAGGCCTCCTTCCGTCGACCT
DS099	AGATCTGGGACCAGCACACTTAGATA
DS100	TCTAGAAGCTCCTATGATAGTCGATA
DS101	AGGCCTATCGCTCCTGTGCTTTTGAA
DS102	AGGCCTATTGGGGTCTTCGAGAAGAC
DS103	GTCGACTAATGCCAACTTTGTACAAG
DS104	ATTGGATACGTTCTCTATGGAGGA
DS105	AAACTCCTCCATAGAGAACGTATC
DS114	CTCGAGTCTTTGAAAAGATAATGTATG
DS115	CTCGAGAGACATAAAAAACAAAAAAAG
DS119	ACTAGTTCTTTGAAAAGATAATGTATG
DS120	ACTAGTAGACATAAAAAACAAAAAAAG
DS122	CTATTCTAGAATGGATAAAGCGGAATTAAT
DS123	ATCGGAATTCTCATAGACCGCGCGTTGATC
DS124	CCCGGGAGCTCCGGATGCAAGGGTTC
DS125	CCCGGGGGTACCGGCCGCAAATTAAAG
DS126	ATGGATAAAGCGGAATTAATTCC
DS127	TCATAGACCGCGCGTTGATCG

Table 2. Overview of plasmids used in this study

Name	Description	Vector type, organism
pCas9F	pvirFpromoter:NLS::Cas9::VirF37C	Expression vector, Agrobacterium
pCas9FPDA1	rightborder:PDA1:KANMX:tef:NLS::Cas9::VirF37C:cyc1t:PDA1:leftborder	Binary vector, yeast
pCas9F2μ	rightborder:KANMX:tef:NLS::Cas9::VirF37C:cyc1t:2μ:leftborder	Binary vector, yeast
psgRNAPDA1	rightborder:PDA1:KANMX:SNR52:gRNACan1:SUP43':PDA1:leftborder	Binary vector, yeast
psgRNA2μ	rightborder:KANMX:SNR52:gRNACan1:SUP43':2μ:leftborder	Binary vector, yeast
pSDM8001	rightborder:PDA1:KANMX:PDA1:leftborder	Binary vector, yeast
pSDM8002	rightborder:KANMX:2μ:leftborder	Binary vector, yeast
psgRNA	pvirFpromoter:SNR52:gRNACan1:SUP4:3'virF	Expression vector, Agrobacterium
p426-SNR52p-gRNA.CAN1.Y-SUP4t	SNR52:gRNACan1:SUP4	Expression vector, yeast

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